**Supplementary Methods:**

*CDC antiviral susceptibility reference virus panels and other viruses displaying reduced drug susceptibility*

Baloxavir susceptibility reference virus panel (version 1.1; International Reagent Resource (IRR); FR-1678) contains five laboratory characterized influenza A viruses, including baloxavir-susceptible and -resistant viruses (with PA-I38L or T or M) was tested. In addition, a set of total 23 influenza A and B viruses with PA substitutions and their sequence-matched control viruses were tested.

NAI susceptibility reference virus panel (version 3.0; IRR; FR-1755) contains eight laboratory characterized influenza A and B viruses, including viruses displaying normal inhibition or reduced (highly) inhibition by one or more NAIs (with NA-H275Y, E119V, D197E, or H134N). Additionally, a set of total 16 influenza A and B viruses with NA substitutions and their sequence-matched control viruses were tested.

*High content imaging-based neutralization test (HINT)*

HINT was performed as previously described (Gubareva et al., 2019). Briefly, for the first step of virus titration, 50 µL MDCK-SIAT1 single-cell suspension was added to wells (0.3x105 cells/well) of a 96-well microplate (black clear-bottom plate, Agilent) containing 100 µL of 10-fold serially diluted (10-1 to 10-7) virus in the absence of TPCK-treated trypsin to achieve single-cycle virus replication. Following 18-24 hpi at 37°C in 5% CO2, cells were fixed with ice-cold methanol:acetic acid (95:5 v/v) and immunostained with mouse anti-NP monoclonal antibody (1:1000; IRR). Cells were then stained simultaneously with goat anti-mouse IgG antibody conjugated to Alexa Fluor-555 (1:1000; ThermoFisher Scientific) and Hoechst 33258 dye (4 µM; AnaSpec Inc.). NP-positive infected cell population (ICP) were detected and quantified using either Celigo (Nexcelom Bioscience) or Cytation 7 (BioTek) image cytometers. The extrapolated virus dilution that would result in ~1000 infected cells per well was used in the inhibition step.

For inhibition, 50 µL of serially diluted antiviral (see Materials and Methods for concentrations) was mixed with 50 µL of virus inoculum in a 96-well microplate, followed by addition of 50 µL MDCK-SIAT1 cell suspension in the absence of TPCK-treated trypsin. Plates were incubated at 37°C in 5% CO2 for 18-24 h. The cells were fixed, stained, and ICP in each well was detected and quantified as described above. EC50s and neutralization titers were calculated by curve-fitting analysis using non-linear regression as previously described (Okomo-Adhiambo et al., 2013, Jorquera et al., 2019).

**Supplementary References**

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